EXHIBIT D

REVIEW ARTICLE

REGULATION AND RECONSTITUTION OF HUMAN HEMATOPOIESIS

John Yu

Abstract: Hematopoiesis is regulated by complex interactions between hematopoietic cells and stromal cells within the bone marrow microenvironment. The stromal cells of this microenvironment secrete hematopoietic factors, produce extracellular matrix and mediate direct cell-to-cell contact; each of these events provides a basis for regulatory control of hematopoiesis. With advances in cell surface phenotyping and cell separation technology, it has been shown that the $CD34^+CD38^-CD45RA^{low}CD71^{low}$ population contains hematopoietic stem cells. Most hematopoietic stem cells are in a quiescent, noncycling state (G_0). In response to external signals, however, they can rapidly enter a functional state (G_1) in preparation for DNA synthesis (S-phase). There have been high expectations, recently, that stimulatory hematopoietic factors will have an important impact on chemotherapy by reducing drug-induced neutropenia and, thereby, allowing dose-intensification of treatment. Similar clinical benefits, however, could also be realized by protecting hematopoietic cells from the cytotoxicity of cycle-specific chemotherapeutic agents by blocking the entrance of the hematopoietic stem cells into the cell cycle. Recent studies have suggested that the addition of a negative regulator can better maintain hematopoiesis *in vitro* and that cells capable of long-term engraftment are primarily comprised of a noncycling population. On the other hand, exposure to cycling-promoting cytokines *ex vivo* may produce an "engraftment defect". An hu-SCID murine model with a high degree of human cell engraftment will be useful for future studies of the *in vivo* effects of various agents, infections or gene therapy on human hematopoiesis.

(J Formos Med Assoc 1996;95:281–93)

Key words:
hematopoiesis
$CD34^+$
marrow microenvironment
stem/progenitor cells
chemotherapy
negative regulator
engraftment
long-term marrow culture initiating cells

Human hematopoietic cells are derived from controlled proliferation and differentiation of hematopoietic stem/progenitor cells [1]. Pluripotent stem cells are capable of both self-renewal and differentiation into specific progenitors. The latter are committed to specific lineages and are functionally defined as colony-forming units (CFUs), eg, progenitors of the erythroid series (burst-forming unit-erythroid, BFU-E and CFU-E), granulocyte/monocyte (CFU-GM) or multipotent CFU (CFU-granulocyte/erythroid/macrophage/megakaryocyte, CFU-GEMM) [1]. Proliferation and differentiation of these stem/progenitor cells are stimulated by specific growth factors, termed "hematopoietic colony-stimulating factors" and interleukins (ILs), which are released by bone marrow stromal cells, as well as hematopoietic accessory cells like monocytes/macrophages or T cells.

Recent advances indicate that human hematopoietic stem cells represent a very minor subpopulation of adult bone marrow (< 1 in 104 to 105 nucleated marrow cells). With the use of multiparameter analysis of cell surface phenotype and cell separation technology, it has been shown that the $CD34^+CD38^-CD45RA^{low}$ and $CD71^{low}$ population contains the hematopoietic stem cells [2, 3]. In addition, human leukocyte antigen-locus DR (HLA-DR)

Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California.
Received: 28 July 1995. Revised: 29 August 1995. Accepted: 17 October 1995.
Reprints and correspondence: to: Dr. John Yu, Department of Molecular and Experimental Medicine, NX 3, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA.

is absent or is expressed at low levels on adult stem cells but is present on fetal or neonatal cells. As in the mouse, Thy-1 antigen is also present on all human hematopoietic stem cells. In the following, new advances in hematopoietic stem cell biology, regulation and reconstitution of human hematopoiesis are discussed.

Human Hematopoietic Stem Cells

Human hematopoietic stem cells express the CD34 antigen, as do the majority of hematopoietic committed progenitors. Enrichment of human hematopoietic stem cells from bone marrow, peripheral blood, or cord blood for transplantation depends predominantly on positive selection of cells using CD34 cell surface molecules. This population of $CD34^+$ cells is very heterogeneous, containing cells that are functionally primitive and capable of giving rise to all hematopoietic lineages, as well as more mature, lineage-specific progenitors. It is conceivable that these selected $CD34^+$ subsets might be more beneficial than unfractionated bone marrow for transplantation as well as for gene therapy. On the other hand, recent studies in mice and humans have identified non-hematopoietic lymphoid cells that function as "facilitator cells" (see Engraftment, "engraftment defects" and "facilitator cells") which enhance allogeneic bone marrow engraftment [4].

CD38 antigen is present on 98% to 99% of all $CD34^+$ hematopoietic cells and absent on hematopoietic stem cells [5, 6]. It has also been suggested that, under single-cell culture conditions, a subset of fetal bone marrow cells with the phenotype $CD34^+CD38^-HLA\text{-}DR^-$ can differentiate into both hematopoietic precursors and stromal cells [6] (however, see recent correction, [7]).

The issue of HLA-DR expression on hematopoietic stem cells warrants further discussion. It was reported that lack of HLA-DR expression or its expression at a low level characterizes cells with extensive *in vitro* expansion capacity and with *in vivo* stem cell function [8, 9]. Furthermore, the increase in HLA-DR expression from cells collected from adult bone marrow and peripheral blood correlates with lineage commitment. On the other hand, it was also reported that HLA-DR is expressed on fetal hematopoietic stem cells [10] and, to a variable extent, on cord blood hematopoietic stem cells. Huang and Terstappen showed that $CD34^+CD38^-$ $HLA\text{-}DR^+$ fraction from fetal bone marrow contains stem cells giving rise to both myeloid and lymphoid precursors [11]. These studies seem to be contradictory with regard to HLA-DR phenotype expression on "hematopoietic stem cells". However, they reflect the difficulty of the unequivocal identification of hematopoietic stem cells on the basis of phenotypic markers alone. Alternatively, the discrepancy among these studies can be attributed to the difference in sample sources (fetal vs adult marrow) [11] or the criteria for quantifying the fluorescence intensity of $HLA\text{-}DR^+$ or $HLA\text{-}DR^{low}$ cells during cell sorting. While most $CD34^+CD38^-$ cells have been reported to express low levels of HLA-DR, the majority of $CD34^+$ HLA-DR^- cells express CD38 [12]. It is, thus, likely that, in both $CD34^+CD38^-$ and $CD34^+$ $HLA\text{-}DR^-$ subsets of potential hematopoietic stem cells, expression of CD38 and HLA-DR on $CD34^+$ bone marrow cells is, to a large extent, mutually exclusive. Another interpretation is that the absence of both CD38 and HLA-DR on $CD34^+$ cells may be used as a marker for hematopoietic stem cells. Alternatively, these phenotypic markers can be two independent variables indicating phenotypic heterogeneity of the hematopoietic stem cell population.

In addition to these markers, Thy-1 at low levels characterizes a subset of $CD34^+$ cells with stem cell function [10]. However, more than 25% of $CD34^+$ cells in adult marrow and blood express $Thy\text{-}1^{low}$ [13], much greater than the predicted incidence of stem cells (< 1% of $CD34^+$ cells) [14]. In addition, cells expressing CD34 and low levels of CD45RA and CD71 are reported to be enriched with multipotent progenitors that proliferate greatly in stroma-containing cultures [115].

Assays for human hematopoietic stem/progenitor cells

Hematopoiesis is analyzed *in vitro* by short- and long-term culture systems (Fig. 1). Short-term marrow

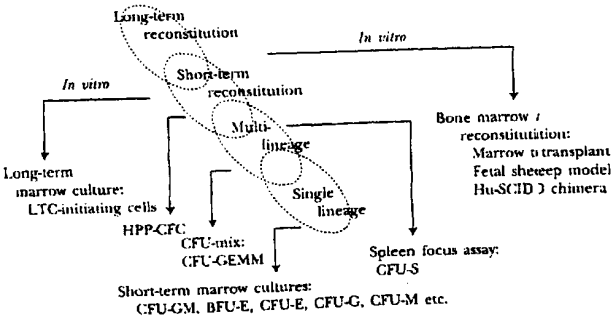

Fig. 1. In vitro *and* in vivo *assays for human hematopoiesis. LTC = long-term marrow culture; Hu-SCID = human-cell reconstituted, severe combined immunodeficient mouse; HPP-CFC = high proliferative potential-colony forming cells; CFU = colony forming unit; CFU-GEMM = ...-granulocyte/erthroid/macrophage/megakaryocyte; CFU-S = ...-spleen; CFU-GM = ...-granulocyte/monocyte; BFU-E = burst-forming unit-erythroid; CFU-E = ...-erthroid series; CFU-G = ...-granulocyte series; CFU-M = ...-megakaryocyte series.*

cultures of different hematopoietic lineages have produced information about committed clonogenic progenitors. In comparison, *in vitro* studies of human hematopoietic stem cells are more difficult because of the complexities of assays. One of the assays available for studies of human hematopoietic stem cells is long-term marrow culture (LTC) [16]. Human LTC has been shown to generate clonogenic progenitors and mature cells in the presence of mesenchymal-derived stromal cells for many weeks [17]. This ability is attributed to the presence and proliferative activity of the so-called "LTC-initiating cells" (LTC-ICs) [18]. Transplantation of LTC-ICs from 10-day-old human marrow LTC into patients treated with lethal chemo-radiotherapy has resulted in a rapid recovery of the hematologic system [19]. In analogous murine LTC, totipotent lympho-myeloid repopulating cells can also be maintained and proliferate over a period of several weeks. Thus, it appears that LTC-ICs are closely related to *in vivo* hematopoietic stem cells with repopulating potential.

LTC-ICs are characterized by their ability to generate clonogenic progenitors (which are assayed with short-term marrow cultures)) after 5 weeks in LTC. The rationale is that clonogenic progenitors present at the beginning of LTC will die or differentiate within 5 weeks, while more primitive stem cells (LTC-ICs) maintain the ability to generate new colony-forming cells. Therefore, analysis of the appearance of the new clonogenic progenitors at week 5, will help in the assessment of the capacity of LTC-IC to maintain *in vitro* hematopoiesis.

Although the number of clonogenic cells present after 5 weeks in LTC provides a quantitative and, hence, useful measure of the LTC-IC frequency in the original population, only relative values are obtained. In order to obtain an absolute measure of these cells, the number of LTC-ICs is determined by a limiting dilution assay. Briefly, mini-LTCs are established in 96-well plates containing preestablished irradiated human stroma or M2-10B4 stromal cells. Cells recovered after 5 weeks from LTC cultures are then plated for LTC-IC determination by limiting dilution assay (cell numbers should be the equivalent of 50 to 800 $CD34^+$ cells at day 0 of culture). For each evaluation, at least three cell concentrations are used with 20 to 24 replicates per concentration. The frequency of negative wells (no clonogenic progenitors detectable 5 weeks later) can be determined; the reciprocal of the concentration of test cells that gives 37% negative cultures will be the fraction of LTC-IC out of all cells initially present (based on the statistical considerations of the poisson distribution for limiting dilutions).

These LTCs have also been useful in the identification of candidate cytokines that may control the proliferation of primitive hematopoietic cells *in vivo*, because these *in vitro* cultures mimic many features of *in vivo* hematopoiesis. For example, primitive hematopoietic cells retained in the adherent layer of an unperturbed LTC are noncycling and in a quiescent state, analogous to the quiescent, noncycling state of human stem cells in bone marrow. However, they can be activated to enter the S-phase of the cell cycle by medium changes, but then return to a quiescent state 4 to 5 days later. This cell cycle oscillation appears to be controlled by a local balance between endogenously produced positive and negative regulators (see below) in the adherent layer of the LTC.

More recently, another *in vitro* technique,, high proliferative potential colony-forming cell (HPP-CFC) culture, has been developed to analyze human hematopoietic stem cells [20]. In addition to LTC, this is the only *in vitro* assay available for studying human hematopoietic stem cells. The HPP-CFCs do not respond to a single growth stimulus but require at least three or more hematopoietic factors for proliferation, generating colonies as large as 3,000 to 8,000 cells per colony. The murine HPP-CFCs were shown to have a highly significant correlation with cells capable of repopulating the bone marrow of lethally irradiated mice. These HPP-CFCs, thus, represent a primitive cell population, that despite being more mature than the LTC-ICs, are closely related to stem cells.

One important feature of the HPP-CFC colonies is the ability to produce additional HPP-CFCs upon replating; thus, these cells have the potential of self-renewal. These experiments demonstrate the presence of cells within primary HPP-CFC colonies capable of forming secondary or tertiary HPP-CFCs. This is an important feature consistent with "self-renewal" of hematopoietic stem cells. Based on the type of secondary colonies formed after replating, three groups of primary HPP-CFC colonies were identified (unpublished observations). The first group (43%) of HPP-CFCs contains cells that form secondary HPP-CFC-derived colonies, in addition to being capable of giving rise to secondary CFU-GM colonies. The second group of colonies (47%) contains cells capable of giving rise to only secondary CFU-GM colonies and the third group consists of fully differentiated progeny unable to sustain the growth of any type of secondary colonies.

Negative regulators of hematopoiesis

Although stimulatory factors are important in the regulation of hematopoiesis, it is generally believed that hematopoietic homeostasis requires the existence of negative regulators, because a hematopoiesis system without such regulators would show wide cyclic variations. Characterization and identification of hematopoietic negative regulators will help to eluci-

date the control of steady-state hematopoiesis. There has been much speculation, in recent years, on the impact that stimulatory hematopoietic factors will have on chemotherapy by reducing drug-induced neutropenia and, thereby, allowing dose-intensification of treatment. Similar clinical benefits, however, could also be realized by protecting hematopoietic cells from the toxic side-effects of cycle-specific chemotherapeutic agents by blocking the entrance of the hematopoietic stem cells into the cell cycle. Therefore, these studies of negative regulators will allow development of new chemoprotectants which protect hematopoietic stem/progenitors from the ravages of chemotherapy and irradiation.

Some putative negative regulators have been reported in the literature. Transforming growth factor-β (TGF-β) is one of the most interesting general negative regulators; it is a member of a large family that shows DNA and amino acid sequence homology. TGF-β is produced by a variety of cells, especially in areas of active hematopoiesis, including the bone marrow and fetal liver. TGF-β specifically inhibits proliferation of stem/early progenitor cells but enhances proliferation of committed late progenitors [21, 22]. TGF-β may have acted directly on progenitor/stem cell growth. Infusion of TGF-β into mice produced a rapid suppression of early stem cell cycling and decreased marrow cellularity and peripheral blood leukopenia [21]. TGF-β induced inhibition can also be neutralized by increasing growth factor concentrations, which indicates a dose-related interaction between positive and negative influences on proliferation of hematopoietic cells [23].

Macrophage inflammatory protein-1α (MIP-1α) is another proliferation inhibitor reported to suppress a number of primitive cells including hematopoietic stem cells. The MIP-1α gene is related to an emerging superfamily of small (8–10 kDa), inducible, secreted proteins, "chemokines" [24, 25], which have proinflammatory and reparative activities [24–26]. Several studies have indicated that MIP-1α is directly inhibitory for spleen colony-forming units (CFU-S) and that administration of MIP-1α can protect primitive murine CFU-S from inactivation by cell cycle-specific drugs *in vivo* [27, 28].

Prostaglandin E is a product of monocytes/macrophages and has a selective suppressor effect on macrophage colony formation, leaving granulocyte proliferation virtually unaltered. Lactoferrin, a product of mature polymorphonuclear leukocytes, has been reported to suppress production of stimulatory factors by some cells but not to have a direct inhibitory effect on granulocyte/macrophage precursors themselves. In addition, other putative specific inhibitors of the hematopoietic stem cells include small regulatory peptides such as AcSDKP [29, 30] and pEEDCK [31]. Injection of either peptides rapidly suppressed CFU-GM cycling andd the recruitment of CFU-S after administration of ara-C [31]. Moreover, both prevented the chemotherapy-induced onset of neutropenia, or at least shortenedd the period of subnormal white blood cell counts.

Although seeveral putative negative regulators have been studied, tthe exact nature, specificity and mechanism of action remain to be defined. LTCs have been particularly useeful in the identification of cytokines that may control tthe proliferation/cycling of primitive hematopoietic cells. Eaves et al had previously identified TGF-β and MIP-1α as the inhibitors that contribute to the proliferation arrrest of primitive cells in the unperturbed LTCs [32]. Reecent data from the same lab, however, appears to rulee out endogenous MIP-1α as a relevant endogenous inhhibitor in this LTC system [32]. They found that thee return to the quiescent state of the hematopoietic cells that occurs 4 to 5 days after medium change cannot be abrogated using an anti-MIP-1α antibody. These reesults imply that the endogenously produced inhibitonr remains unidentified.

Interactionas within the marrow microenvironmentt

Human hematopoiesis is regulated by complex interactions among hematopoietic cells and stromal cells within the bonne marrow microenvironment (Fig. 2). The stromal cells of this microenvironment represent a heeterogenous population, consisting of fibroblasts, enndothelial cells, adipocytes, osteoclasts and monocytes/macrophages. They secrete cytokines, produce extraccellular matrix and mediate direct cell-to-cell contact;; each of these events provides a basis for regulatory control of hematopoiesis.

Although onnly cytokines are required for the proliferation/differeentiation of hematopoietic progenitors in *in vitro*, shoort-term clonogenic assays, long-term cultures cannoot be sustained over months unless an adherent strommal layer and the extracellular matrix are present in the cultures. Indeed, hematopoiesis in the marrow midcroenvironment requires close association of hematoppoietic stem/progenitor cells with both marrow stromaal cells and the extracellular matrix. The importance of similar cell-to-substrate or direct cell-to-cell contacts haas been well studied in lymphoid systems [33]. In contraast, the role of adhesion molecules and the extracellulaar matrix, or both, in the regulation of hematopoiesis is just beginning to be elucidated.

Figure 2 shcows a schematic representation of this complex stromna-hematopoietic interaction. Purified human $CD34^+$ cells express very late antigen-4 (VLA-4), VLA-5, and at least one or more β_2 integrins [34, 35]. Human maarrow stromal cells express vascular cell adhesion moldecule-1 (VCAM-1) and fibronectin

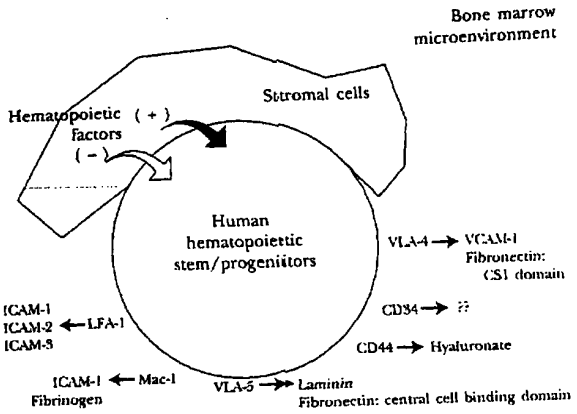

Fig. 2. *Dynamic interactions within the bone marrow microenvironment. VLA = very late antigen; LFA = leukocyte function antigen; VCAM = vascular cell adhesion molecule; ICAM = intercellular adhesion molecule.*

(ligands for VLA-4 and VLA-5) as well as intercellular adhesion molecule-1 (ICAM-1) (ligand for the β_2 integrins, LFA-1 and Mac-1l) [34]. In addition to adhesion molecules, extracellullar matrix proteins such as fibronectin, collagens, hemonectin, thrombospondin, proteoglycans and glycosaminoglycans [35] also provide anchorage sites for both hematopoietic cells and cytokines in the marrow microenvironment [36, 37]. Fibronectin, a prototype ligand for stromahematopoietic interactions, is composed of A and B chains that are linked by two disulfide bonds. A central cell-binding region of fibronectiin, which includes the amino acid sequence Arg-Gly-Asp-(Ser) [RGD(S)], is recognized by VLA-5 ($\alpha_5\beta_1$) [38, 39] and is important for the binding of hematopoietic progenitors (see below). A second site, the CS1-containing fragment, located near the C-terminal heparin-binding domain and contained within an alternatively spliced region, is recognized by the integrin VLA-4 ($\alpha_4\beta_1$) [40, 41].

Patel and Lodish have shown that BFU-E and more mature erythroid precursors adhere to the central cell-binding domain of fibronectin via the integrin $\alpha_5\beta_1$ [42]. This conclusion was suggested by the specific inhibition of the interaction by RGD-containing peptides [42] and by monoclonal antibodies directed against either the RGD-containing cell-binding domain of fibronectin [43] or VLA-5 [44]. Another study, however, suggested the involvement of VLA-4 ($\alpha_4\beta_1$) in the adhesion of human erythroblastic progenitors to the CS1-site of fibronectim [45]. A recent report further showed that the interactions between VLA-4 and VCAM-1 were involved in the formation of erythroblastic islands during developmemt of erythropoiesis [46].

On the other hand, more primitive hematopoietic cells such as murine day-12 CFU-S or the human $CD34^+DR^-$ bone marrow cells adhered to an isolated CS-1 fragment of fibronectin [47]. It was also shown that the α_4 subunit of the VLA-4 integrin was expressed on day-12 CFU-S. Furthermore, infusion of anti-VLA-4 antibodies into primates led to rapid "peripheralization" of hematopoietic progenitors into blood [48].. In the study by Williams et al, however, the day-12 CFU-S cells did not adhere to intact fibronectin [47]. It appears that the stroma-hematopoietic interactions may be more complex than originally envisioned. Verfaillie et al reported that primitive LTC-IC and CFU-GEMM progenitors adhere to the 33/66 kD C-terminal heparin-binding fragment of fibronectin; in contrast,, more-differentiated, committed progenitors adhere equally well to both the 33/66 kD and the 75 kD RGD-dependent fragment [49]. In the 33/66 kD fragment, there are three sites known for cell-attachment: fibronectiin-C/H I, fibronectin-C/H II and CS1 fragments [49]. LTC-IC and CFU-GEMM progenitors adhere significantly better to fibronectin-C/H II than to the two flanking sites [49].

Recent studies also indicate that the attachment of human $CD34^+$ cells to a marrow-derived stromal layer *in vitro* involves VLA-4/VCAM-1, VLA-5/fibronectin, and β_2-integrin/ICAM-1 [34, 50]. $CD34^+$ cells seem to use VLA-5, and to a lesser extent VLA-4, to adhere to fibronectin. However, this binding, which could be inhibited by specific antibodies and peptides [34, 50], took place only after activation of the $CD34^+$ cells with cytokines (see below). It should be noted that antibodies to VLA-4α, β_1, other VLA integrins, or VCAM-1 never completely inhibit cell adhesion to marrow stroma [35]. This suggests a role for other classes of integrins or other types of adhesion receptors in stroma-hematopoietic interactions. Verfaillie et al found that adhesion to fibronectin is mediated by proteoglycans and by the CD44 adhesion molecule, in addition to VLA-4 [51].

Dynamic regulation of stroma-hematopoietic interactions

In addition to cytokines, hematopoiesis is controlled by interactions between adhesion molecules and their ligands in the surrounding extracellular matrix. The cell adhesiveness can be altered by modifying biosynthetic patterns, expression on the cell surface, mRNA splicing and/or posttranslational modifications of these adhesion/cell matrix proteins [52]. Another unique feature is a rapid and reversible modulation of receptor function observed for the integrin family [52]. Cells can rapidly alter the binding affinity of the integrins for their ligands. This "inside-out signalling" has been shown to be a property of β_1, β_2, and $\alpha_{IIb}\beta_3$ integrins in many other systems [53]. Besides adhesion molecules and extracellular matrix proteins, it is anticipated that some "anchored" growth fac-

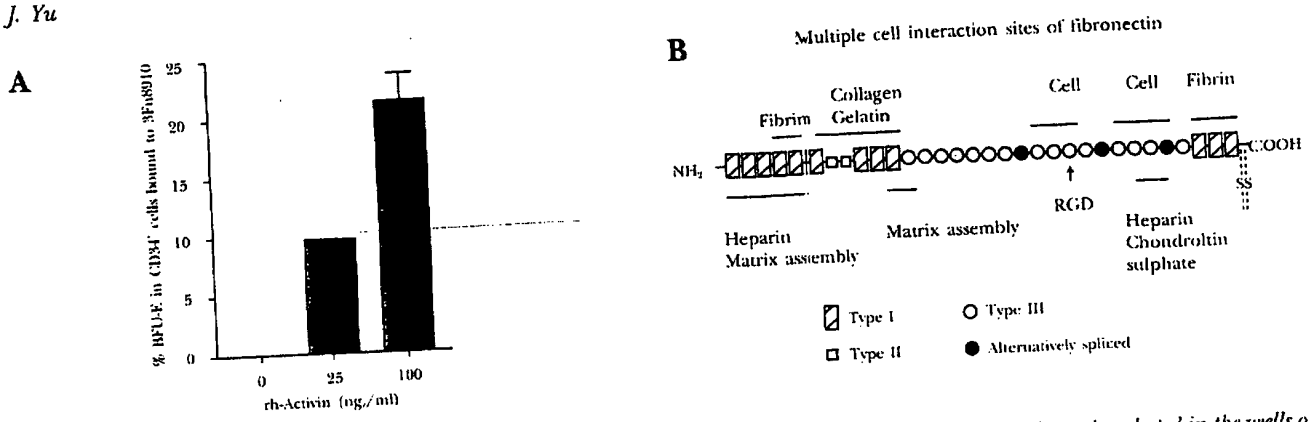

Fig. 3. *Enhanced cytoadhesion of BFU-E from CD34⁺ cells to recombinant 3Fn8910. A) Recombinant 3Fn8910 was incubated in the wells of microtiter plate for 2 h at 37°C. The wells were first blocked with 1% BSA; and purified human CD34⁺ cells were preincubated in ibuffer containing 0, 25 and 100 ng/mL of recombinant hybrid (rh)-activin A for 30 min and then transferred to the 3Fn8910-coated plates. After incubation for 1 h at 37°C, the nonadherent cells were washed off with gentle pipetting. The residual adherent cells were then cultured for BFU-E and CFU-GM in methylcellulose. B) The modular structure of fibronectin, showing the position of type I, II and II modules. Fibronectin residues 1235–1510 were fused to maltose-binding protein and expressed as recombinant hybrid protein, 3Fn8910, containing the 8, 9 and 10th type III repeats. It represents the central cell-binding domain which contributes to integrin $\alpha_5\beta_1$ binding.*

tors such as membrane-bound basic fibroblast growth factor, monocyte colony stimulating factor or stem cell factor (SCF) may participate in the adhesion process.

Currently, it is known that VCAM-1 in human umbilical vein endothelium can be up-regulated by treatment with tumor necrosis factor (TNF)-α, IL-1 or lipopolysaccharide [54]. Similarly, expression of VCAM-1 by human stromal cells is increased with IL-1, IL-4, or TNF-α stimulation [34, 50]. Treatment with interferon-γ or phorbol myristate acetate rapidly enhanced the level of ICAM-1 and prolonged its half-life at the cell surface [55].

Modulation of the avidity of integrins such as $\alpha_4\beta_1$ and $\alpha_5\beta_1$ was observed after treatment with SCF, whereas the expression of these molecules at the cell surface was little affected [56]. Analogous affinity changes of integrins had been reported for the regulation of β_1 integrin function by monoclonal antibodies to this β_1 subunit [57] and also by antibodies to platelet endothelial cell adhesion molecule-1 (PECAM-1) [58]. It has also been shown that in the T cell system, β_1 affinity can be modulated by ligation of T cell receptors [59] and β_2 affinity can also be modulated in the same way [60]. In contrast, neutrophil Mac-1 (CD11b/CD18) and MEL-14 adhesion proteins are inversely regulated by chemotatic factors [61]. These studies strongly suggest the importance of the inside-out signaling, unique to integrin receptors.

TGF-β is known to play an important role not only in synthesis and degradation of matrix proteins but also in the expression of integrin receptors [62]. A preliminary study found a related protein, activin A [63], to have profound influences on the adhesive properties of hematopoietic cells toward recominbant fibronectin fragment 3Fn8910 (Fig. 3A), suggesting the possibility of modulation of integrin activities in these cells. The recombinant hybrid protein fused to maltose-binding protein in the expression plasmid pIH821 [64] contains the 8th, 9th and 10th type III repeats of fibronectin (Fig. 3B), representing the central region of fibronectin that contributes to integrin $\alpha_5\beta_1$ binding.

Cytokine-dependent expansion of hematopoiesis *ex vivo*

Cytokine-supplemented *ex vivo* expansion has generated tremendous enthusiasm in recent years. Transplantation of 5-fluorouracil-treated mouse marrow expanded for 7 days with IL-1, IL-6 and SCF, resulted in greatly accelerated recovery of blood neutrophils, platelets and hematocrit compared to mice receiving uncultured marrow [65]. This acute survival-promoting action of expanded marrow can be attributed to the > 500-fold increase in CFU-GM, > 120-fold increase in CFU-S, and 80-fold increase in HPP-CFCs seen over the 7 days of culture. *Ex vivo* expansion of human CD34⁺ cells is achieved in serial delta cultures with progenitor cell expansions of up to 75-fold in 2 weeks with combinations of IL-1 + SCF + IL-3 + IL-6 or SCF + IL-6 + IL-3 + G-CSF + erythropoietin [66].

However, the controversial issue is the degree to which human stem cells are maintained or expanded in these cultures. The consensus is that these stroma-free, cytokine-supplemented cultures result in large mature cell expansion and moderate (yet variable)

progenitor cell expansion, but the number of LTC-ICs often declines to below the input level. In this context, it should be noted that the addition of the negative regulator, MIP-1α plus IL-3 to "stroma-noncontact" culture systems significantly promoted the maintenance of LTC-ICs in culture (see below). Moreover, the source of hematopoietic stem cells may be important: 15- to 20-fold expansion of LTC-ICs was seen in similar cytokine-driven cultures of cord blood $CD34^+$ cells at 1 and 2 weeks [67].

In contrast to *ex vivo* liquid expansion, the LTC system contains stromal cells, which contribute both stimulatory and inhibitory signals on interaction with hematopoietic cells. Direct comparison of stromal cultures with stroma-free *ex vivo* expansions indicates a role for stroma in the stimulation of adult human stem cells that cannot be fully duplicated by the soluble cytokines, with stromal systems generating fivefold more LTC-ICs after culture [68]. These and other experiments indicate the importance of unidentified factors from marrow stromal layers [67, 68].

The role of marrow stroma is more complex than originally anticipated. Verfaillie's group reported that in "stroma-noncontact" cultures, up to 50% of LTC-ICs can be recovered by 5 weeks and 100% maintenance is obtained over 8 weeks if cultures are supplemented with IL-3 and MIP-1α, as well as undefined diffusable factors from stroma [67]. While IL-3 is a growth-promoting cytokine and MIP-1α is a known negative hematopoietic regulator, the mechanisms through which this cytokine combination maintains primitive stem cells are unclear. On one hand, separation of human stem cells from stroma may facilitate proliferation of human stem cells by removal of the cells from local negative influences. On the other hand, because addition of MIP-1α without the growth-promoting cytokine IL-3 failed to increase the recovery of LTC-ICs, MIP-1α may not have a direct proliferation-inducing effect on human stem cells [67]. Instead, MIP-1α most likely prevents the terminal differentiation of immature progenitors induced to proliferate by relatively high levels of growth-promoting factors such as IL-3 and, thereby, facilitates the expansion and accumulation, or both, of primitive hematopoietic cells.

Moreover, when "stroma-free" cultures were compared with "stroma-noncontact" cultures, the same combinations of cytokines resulted in only 5% recovery of LTC-ICs. These experiments, therefore, also indicate that additional stroma-derived soluble factors, not yet identified, are synergyzing with MIP-1α + IL-3 to maintain a constant level of human LTC-ICs in *in vitro* cultures. As MIP-1α receptors are present on monocytes/macrophages, the aforementioned observations could be the result of the induction of secondary factors produced by stromal macrophages. Alternatively, MIP-1α plus IL-3 may exert effects through direct interaction with hematopoietic cells rather than through altering the marrow microenvironment milieu.

Engraftment, "engraftment defects" and "facilitator cells"

The bone marrow microenvironment is generally believed to render both stimulatory and inhibitory activities toward hematopoietic stem cells. It was reported that there are cell cycle and functional differences between $CD34^+/CD38^{high}$ and $CD34^+/CD38^{low}$ human marrow cells after *in vitro* cytokine exposure [69]. This is consistent with the findings of Fleming et al indicating that murine stem cells ($Thy1.1^{low}Lin^{-/low}Sca\text{-}1^+$) can be divided into resting and actively proliferating cells [70].

More important is the finding that injection of noncycling stem cells (G_0/G_1) rescued 90% of lethally irradiated mice, whereas injection of the actively cycling $S/G_2/M$ cells rescued only 25% [70]. Whether the decreased reconstitution capacity of the cycling stem cells is the result of a loss of their differentiation potential/self-renewal capacity, or is due to a defect in the homing of these cells to a favorable microenvironment remains to be determined. However, a decreased long-term reconstitution capacity of the $S/G_2/M$ subset was observed when these cells were coinjected with a radioprotective dose of syngeneic bone marrow. These data are consistent with two hypotheses: 1) the $S/G_2/M$ cycling stem cells differentiate into the committed progenitor cell pool, or alternatively, 2) they may retain stem cell functionality but have a reduced capacity for engraftment on a per cell basis.

Given that hematopoietic cells responsible for early engraftment consist of cells that are highly proliferative and short-lived, while cells capable of long-term engraftment are comprised primarily of a noncycling population, it is reasonable to suggest that the actively proliferating and resting cells may represent compartments of human hematopoietic cells responsible for rapid and long-term bone marrow engraftment.

Delineation of the signals regulating cycling/noncycling stem cells and their relative hematopoietic engraftment potential awaits further investigation. In Abkowitz's laboratory, glucose-6-phosphate dehydrogengse (G6PD) phenotypes of BFU-E and CFU-GM were repeatedly assayed for 4 to 6 years after transplantation in heterozygous cats in order to track the contributions of stem cell clones to the progenitor cell compartment [71]. Initially, significant fluctuations were seen in the G6PD phenotype of progenitor cells, implying variable contributions of stem cell clones. Only after as long as 1 to 4.5 years did clonal contributions to hematopoiesis stabilize, suggesting that

hematopoiesis can be stably maintained by the progeny of one (or a few) cells [71]. These results indicate that the individual stem cell has a vast self-renewal and proliferative capacity, or both. Computer simulation further suggests that the transplanted "stem cells" are mostly dormant, having a probability of replication of 1 per 12 weeks (Abkowitz, personal communication).

Quesenberry et al compared engraftment of male murine marrow cells after *ex vivo* cytokine exposure (IL-3 + IL-6 + IL-11 + SCF) to the volume equivalent of the starting noncultured cells in female syngeneic recipients [72]. It was found that noncultured marrow cells resulted in more than 20% engraftment while cytokine-exposed cultured marrow showed no evidence of engraftment. These data indicate that exposure of marrow cells to stimulatory cytokines induced cell cycle activation and expansion of progenitor stem cells, while at the same time producing their profound engraftment defect. Thus, the finding agrees with other studies indicating that cells which engraft in the hosts are quiescent or dormant and not in an active cell cycle. It is further hypothesized that the defects seen with cytokine-exposed marrow [72] and with post-5 fluorouracil marrow [73] may be related to the cell cycle status of these cells such that their progression through cell cycling diminishes the engraftment capacity of hematopoietic cells [72].

Graft-vs-host disease (GVHD) and failure of engraftment continue to cause significant morbidity and mortality, and constitute current challenges in bone marrow transplantation as well as the humanized immunodeficient mouse model (see below). Recent experimental (mouse) and clinical studies have evaluated the benefit of utilizing purified hematopoietic progenitors/stem cells as hematopoietic graft cells for transplantation. However, studies in experimental rodent models and clinical data [74, 75] have shown that pan-T cell depletion results in decreased incidence of fatal GVHD, but at the cost of increased failure of allogeneic-hematopoietic engraftment [76]. The *in vivo* studies in the mouse system by Kaufman et al [4] suggests that a population of lymphoid cells ($CD8^+CD45R^+\alpha\beta TCR^-$), so called "facilitator cells" isolated from the characteristic lymphoid gate during cell sorting, increase allogeneic engraftment when cotransplanted with major histocompatibility complex-matched hematopoietic stem cells [4]. A similar phenomena was also seen in the human *in vitro* studies done by Schmidt-Wolf et al [77]. They reported that the presence of human $CD4^-CD8^-TCR^+$ T cells, and also the so-called "natural suppressor", suppress the mixed leukocyte reaction and have been postulated to lower the risk of GVHD [77]. Recent studies strongly suggest that primates, including humans, contain "facilitating cells" which can be isolated using three-color sorting techniques from the lymphoid region. Given that the transplanted cells capable of long-term engraftment primarily comprise a noncycling population [70], it is tempting to speculate that the facilitating cells function by "embracing" hematopoietic stem cells with negative regulatory activities and, thus, prevent these stem cells from continous proliferation in favor of engraftment. As accessory cells might increase the chance for engraftment, attempts to enrich stem cells might remove these putative "facilitator cells."

Human-mouse chimeras as models for human hematopoiesis

Many of the aforementioned studies are *in vitro* assays. Our understanding of the biologic effects of various agents, infections or consequences of gene therapy on human hematopoiesis is incomplete because of the lack of *in vivo* human stem cell assays, other than clinical bone marrow transplants. As a small animal model, the human-cell reconstituted, severe combined immunodeficient (hu-SCID) mouse has been useful for studying human disease [78–80] and in some cases as a model for gene therapy [81].

Several approaches for engrafting human hematopoietic cells into immunodeficient mice have been described. They employ transplantation of adult bone marrow, mature lymphoid cells or fetal organs to SCID mice [78–80]. However, these mouse models have been of limited use for hematopoietic studies, because engraftment is very low and mainly with macrophage or lymphoid lineages [78–80]. This obstacle of low human hematopoietic cell engraftment was first overcome by Lapidot et al [82] who demonstrated that irradiated adult SCID mice with whole bone marrow supplemented with large doses of the human growth factors such as erythropoietin, SCF and PIXY321, allowed human hematopoietic cells to engraft. Most important, however, was the observation that the mice developed both multilineage and committed myeloid and erythroid progenitors [[82]. Human cells were found in the mouse bone marrow, spleen and liver. The suggestion that hu-SCID mice might engraft primitive progenitors has, thus, been greeted with enthusiasm.

Although the hu-SCID model is adequate, we would like to improve on it so as to have self-sustaining multilineage hematopoiesis, or at the minimum, increased human cell levels and engraftment of primitive progenitors. In addition to using neonatal SCID mice as recipients of human tissue [83], we have recently taken steps to improve human cell engraftment. Firstly, in collaboration with Dr. D. Mosier at the Scripps Institute, , our laboratory has various immunodeficient mice, developed by selective breeding of genetically altered mice, which lack various host components that could hinder xenoge-

engraftment of bone marrow components. This new generation of T and B cell immunodeficient mice have decreased function and reduced levels of mouse natural killer (NK) cells and macrophages. They include SCID.CID (SCID mice x mouse class I deficient mice), RAG II KO.CID (recombinase II knockout mice x CID mice), RAG II KO.Prf. (RAG II KO mice x perforin knockout mice) and NOD.SCID (nonobese diabetic mice x SCID mice).

Recently, human $CD34^+$ cells, rather than whole bone marrow cells are now used for hu-SCID studies, in order to enrich the ratio of target cells for engraftment and reduce graft versus host disease seen when bone marrow is used for reconstitution of neonatal SCID mice [84] and also to provide a self-sustaining system. Finally, another issue related to the establishment of hu-SCID models using purified hematopoietic stem cells is the changes in hematopoietic phenotypes or development of NK activities after incubation with cytokines (also see Section 7). It has been reported that the phenotypes of hematopoietic precursors can be modulated by cytokines during *in vitro* manipulation. Interferon γ but not IL-4 may significantly increase the expression of CD38 on $CD34^+$ cells, whereas both cytokines may act additively to increase HLA-DR expression [12]. It was also shown that $CD34^+CD33^-$ or $CD34^+CD38^-$ cells can be induced to express functional NK cell activity in the presence of marrow stroma and IL-2 [8]. Therefore, phenotypic characteristics of $CD34^+$ after suspension cultures (eg, activation for retrooviral transduction) should be monitored for any indication of phenotypic changes.

In collaboration with Dr. B. Torbett, our laboratory developed the SCID mouse model for a high level of engraftment of human multilineagic hematopoiesis. As shown in Figure 4, we achieved reconstitution of irradiated SCID mice with cord blood-derived mononuclear cells (MNCs), as well as with $CD4^+CD3^+CD20^+$-depleted preparations. Figure 4 demonstrates that a representative hu-SCID mouse #424 has HLA, $CD14^+$, $CD2^+$, $CD20^+$, $CD45^+$ and $CD34^+$ cells at 6 weeks. The percentage of human cells are also shown in the legend to Figure 4. More significant is the finding that human cells are engrafted mostly in bone marrow but not in spleen.

To determine whether the $CD14^+$ cells in the SSCID mice were derived from primitive or committed human progenitors, we assessed whether human CFU-GM were present. We demonstrated that these progenitors engrafted in hu-SCID mice give rise to human CFU-GM (88 ± 11/plate) and BFU-E (26 ± 8/plate). Thus, cells of the myeloid and erythroid lineage seen by flow

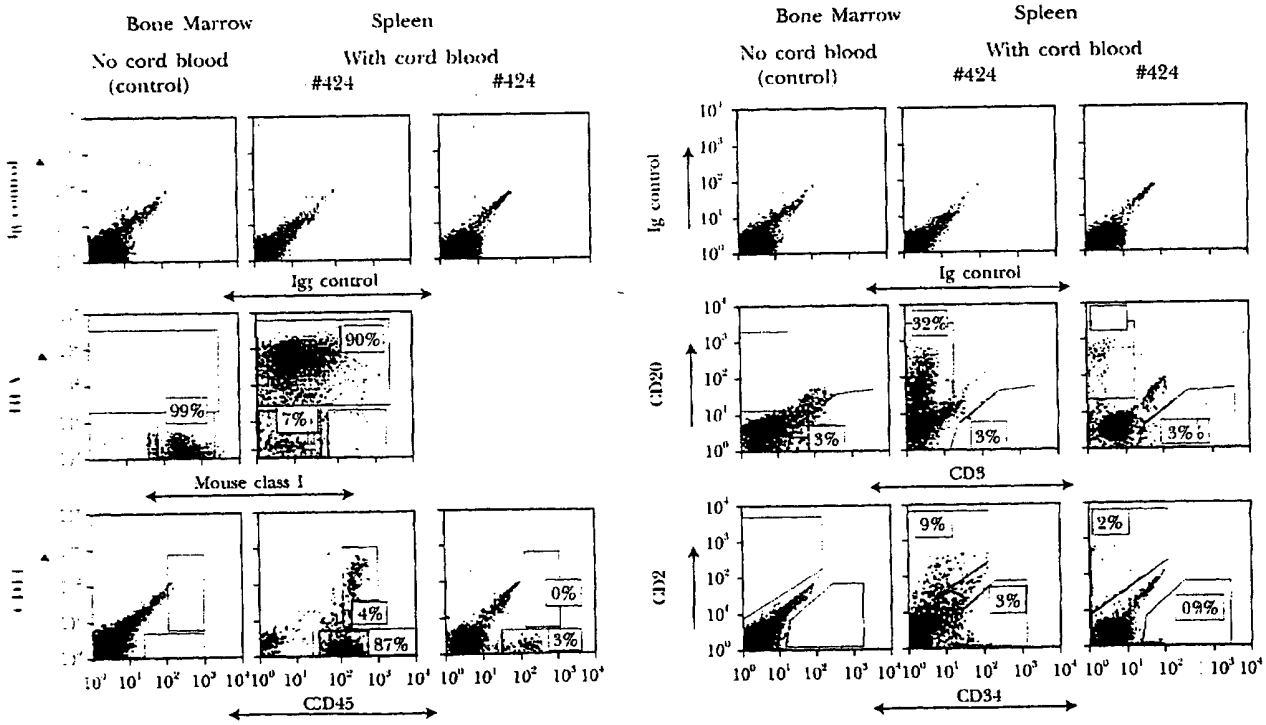

Fig. 4. Human hematopoietic cells are evident in adult SCID mouse bone marrow. Irradiated SCID mice intravenously transplanted with 2×10^7 cord blood cells demonstrate HLA^+ (90%), $CD2^+$ (9%), $CD14^+$ (4%), $CD20^+$ (32%), $CD34^+$ (3%), and $CD45^+$ (87%) cells from whole bone marrow 6 weeks after engraftment. These cells also demonstrated committed progenitors as shown by BFU-E and CFU-GM colonies in a standard clonogenic assay.

cytometric analyses are developing from human committed progenitors present in the $CD34^+$ population, which engrafted in hu-SCID mice (Fig. 4).

$CD20^+$ B cells could also be demonstrated in the hu-SCID mice, suggesting that B cell progenitors are present. An alternative explanation is that $CD20^+$ cells present in the cord blood preparations were simply expanding in the hu-SCID mouse. To disprove the latter possibility, SCID mice were reconstituted with a $CD3^+CD4^-CD20^-$-depleted population. To control for input of the number of $CD34^+$ cells into each SCID mouse, the total numbers of human cells were adjusted so that each SCID mouse received approximately 3×10^5 $CD34^+CD33^-$ cells. The results comparing hu-SCID mice reconstituted with total cord blood-derived MNC or with a $CD3^+CD4^-CD20^+$-depleted subpopulation at 6 weeks revealed that the total numbers of human cells and $CD20^+$ cells were found in similar numbers in hu-SCID mice whether xenografts were depleted of $CD3^+CD4^+CD20^+$ cells or not. Total numbers of $CD45^+$ and $CD33^+$ cells were also similar between hu-SCID mice regardless of total cell input. These results suggest that human cells in the mice are derived from the engrafted $CD34^+$ cell population. Thus, our data are consistent with engraftment of primitive or committed progenitors in hu-SCID mice capable of giving rise to myeloid, lymphoid and erythroid lineages.

The presence of human phenotypes and the levels of human clonogenic progenitors provide information with respect to the possibility of engraftment. There will, however, be the issue of long-term versus short-term engraftment after transplantation. The longer the time we detect committed progenitors after reconstitution, the more suggestive it is that hematopoietic stem/progenitor cells have engrafted. The shorter the time committed progenitors are found, the more suggestive it is of little or no engraftment, loss or rapid differentiation of hematopoietic cells.

Although prolonged clonogenic progenitor production is suggestive of hematopoietic stem cell engraftment, it is not conclusive. It is possible that in the transplanted SCID mice only committed progenitors engraft. This would be analogous to hu-SCID mice where only mature lymphoid cells remain [78]. Alternatively, hematopoietic stem/progenitors cells are present but dormant [71], giving rise to committed progenitors upon activation. This last possibility has been suggested by Lapidot et al [82] who found CFU-GM/BFU-E activity after resuming human growth factor treatment of "rested" SCID mice, and by Nolta et al [85] who found $CD45^+$ cells and CFU-GM/BFU-E activity 9 months post reconstitution. Therefore, a more stringent test for engraftment is the success of serial transfer from a hu-SCID mouse to another SCID mouse. This rationale is based on the finding that rescue of hematopoietic function with hematopoietic stem/progenitor cells is more sensitive than any *in vitro* assay.

Concluding Remarks

Although only soluble hematopoietic factors are required for the *in vitro* proliferation/differentiation of hematopoietic progenitors in short-term colony assays, hematopoiesis in the marrow microenvironment requires close interaction between hematopoietic stem/progenitor cells and marrow stromal cells and with extracellular matrix. Although there are high expectations that stimulatory hematopoietic factors and highly purified "stem cells" will prove to be clinically important, the possibility of "engraftment defect" as a result of cytokine exposure and the existence of putative "facilitator cells" await further studies. In addition to the stimulatory factors, negative regulators may also be important for the maintenance and engraftment of human hematopoietic cells. However, their identity, specificity and mechanism of action remain to be delineated. Particularly intriguing are the findings that cells which engraft in the hosts are quiescent or dormant and not in active cell cycle. In light of the current enthusiasm for transplantation medicine and gene therapy, the signals regulating cycling/noncycling hematopoietic stem cells warrant detailed investigation.

ACKNOWLEDGMENTS: This manuscript is dedicated to the memory of the late alumnus Dr. Alan Ming-ta Wu (NTU, 1964). Prior to the advent of recombinant erythropoietin, Dr. Wu shipped two precious ampules of erythropoietin, purified from urine, from Canada to us so that we could perform our first marrow cultures. In 1980, at an international meeting in Canada, Dr. Wu revealed to me two secrets: he was sufferring from astrocytoma and he was serving as Chairman of the then outlawed World United Formosans for Independence, Canada Headquarters. In 1982, Dr. Wu passed away at the age of 43. Many of his colleagues, including, Drs. Donald Metcalf, Robert C. Gallo, Connie J. Eaves and Arthur A. Axelrad, have become leaders in the field. The work described in this article was supported by grants DK40218, DK49886 and MO1-RR00833 from the National Institutes of Health. This is publication number 9429-MEM from The Scripps Research Institute.

References

1. Metcalf D: The molecular control of cell division, differentiation, commitment and maturation in haemopoietic cells. *Nature* 1989;339:27–30.
2. Baum CM, Weissman IL, Tsukamoto AS, et al: Isolation of a candidate human hematopoietic stem-cell popula-

tion. *Proc Natl Acad Sci USA* 1992;89:2804–8.
3. Terstappen LW, Huang S, Safford M, et al: Sequential generations of hematopoietic colonies derived from single nonlineage-committeed CD34+CD38– progenitor cells. *Blood* 1991;77:1218–27.
4. Kaufman CL, Colson YL, Wren SM, et al: Phenotypic characterization of a novel bone marrow derived cell that facilitates engraftment of allogeneic bone marrow stem cells. *Blood* 1994;84:2436–46. (In Press)
5. Issaad C, Croisille L, Katz A, et al: A murine stromal cell line allows the proliferation of very primitive human CD34+/CD38– progenitor cells in long-term cultures and semisolid assays. *Blood* 1993;81:2916–24.
6. Huang S, Terstappen LW: Formation of hemato-poietic microenvironment and hematopoietic stem cells from single human bone marrow stem cells. *Nature* 1992;360:745–9.
7. Huang S, Terstappen LW: Formation of haematopoietic microenvironment and haematopoietic stem cells from single human bone marrow stem cells [retraction of Huang S, Terstappen LW. *Nature*, 1992, Dec 24-31;360(6406):745–9]. *Nature* 1994;368:664.
8. Miller JS, Verfaillie C, McGlave P: The generation of human natural killer cells from CD34+/DR-primitive progenitors in long-term bone marrow culture. *Blood* 1992;80:2182–7.
9. Srour EF, Zanjani ED, Cornetta K, et al: Persistence of human multilineage, self-renewing lymphohematopoietic stem cells in chimeric sheep. *Blood* 1993;82:3333–42.
10. Peault B, Weissman IL, Buckle AM, et al: Thy-1 expressing CD34+ human cells express multiple hematopoietic potentialities *in vitro* and in SCID-hu mice. *Nouv Rev Fr Hematol* 1993;35:91–3.
11. Huang S, Terstappen LW: Lymphoid and myeloid differentiation of single human CD34+, HLA-DR+, CD38– hematopoietic stem cells. *Blood* 1994;83:1515–26.
12. Snoeck H-W, Lardon F, Lenjou M, et al: Differential regulation of the expression of CD38 and human leukocyte antigen-DR on CD34+ hematopoietic progenitor cells by interleukin-4 and interferon-gamma. *Exp Hematol* 1993;21:1480–6.
13. Craig W, Kay R, Cutler RL, et al: Expression of Thy-1 on human hematopoietic progenitor cells. *J Exp Med* 1993;177:1331–42.
14. Goldstein NI, Moore MAS, Allen C, et al: A human fetal spleen cell line, immortalized with SV40 T-antigen, will support the growth of CD34+ long-term culture-initiating cells. *Mol Cell Differ* 1993;1:301–21.
15. Mayani H, Dragowska W, Lansdorp PM: Characterization of functionally distinct subpopulations of CD34+ cord blood cells in serum-free long-term cultures supplemented with hematopoietic cytokines. *Blood* 1993;82: 2664–72.
16. Eaves CJ, Cashman JD, Eaves AC: Methodology of long-term culture of human hemopoietic cells. *J Tissue Culture Methods on Stem Cell Biol* 1991;13:55–62.
17. Sutherland HJ, Lansdorp PM, Henkelman DH, et al: Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *Proc Natl Acad Sci USA* 1990;8787:3584–8.
18. Sutherlerland HJ, Eaves CJ, Eaves AC, et al: Charaacterization anand partial purification of human marroow cells capableole of initiating long-term hematopoiesis *i in vitro*. *Blood* 1989;74:1563–70.
19. Barnettett MJ, Eaves CJ, Phillips GL, et al: Successful autograrafting in chronic myeloid leukaemia after maintenance e of marrow in culture. *Bone Marrow Traansplant* 1989;4:4:345–51.
20. McNiecece IK, Stewart FM, Deacon DM, et al: Deteection of a humanan CFC with a high proliferative potentiaal. *Blood* 1989;7474:609–12.
21. Ruscettetti FW, Jacobsen SE, Birchenall-Roberts MM, et al: Role of of transforming growth factor-b1 in regulaation of hematotopoiesis. *Ann NY Acad Sci* 1991;628:31–43.
22. Sargiacacomo M, Valtieri M, Gabbianelli M, et aal: Pure human in hematopoietic progenitors: direct inhibitory effect of of transforming growth factors-b1 and -b2. *Ann NY Acad ScSci* 1991;628:84–91.
23. Bradleyey TR, Millar JL, Bertoncello I, et al: Negativee regulators of hf hematopoiesis. *Ann NY Acad Sci* 1991;628:582–8.
24. Oppenheim JJ, Zachariae CO, Mukaida N, et al: Properties of the novel proinflammatory supergene "inteercrine" cytokinine family. *Annu Rev Immunol* 1991;9:617–448.
25. Wolpe e SD, Cerami A: Macrophage inflammatoory proteins 1 al and 2: members of a novel superfamily of cyytokines. *FASEB JB J* 1989;3:2565–73.
26. Baggioliolini M, Dahinden CA: CC chemokines in allergic inflammmmation. *Immunol Today* 1994;15:127–33.
27. Dunlopop DJ, Wright EG, Lorimore S, et al: Demonsstration of stemm cell inhibition and myeloprotective effffects of SCI/rhrhMIP1 alpha *in vivo*. *Blood* 1992;79:2221–55.
28. Lord B BI, Dexter TM, Clements JM, et al: Macrcophage-inflammmmatory protein protects multipotent hematcopoietic cells frfrom the cytotoxic effects of hydroxyurea . *in vivo*. *Blood* 1992;79:2605–9.
29. Frindellel E, Guigon M: Inhibition of CFU entry innto cycle by a bopone marrow extract. *Exp Hematol* 1977;5:744–6.
30. Bogdelen AE, Carde P, Deschamps de Paillette FE, et al: Ameliolioration of chemotherapy-induced toxiicity by cotreatiatment with AcSDKP, a tetrapeptide inhiibitor of hematotopoietic stem cell proliferation. *Ann NY Acad Sci* 1991;62628:126–39.
31. Paukovovits WR, Moser M-H, Rutter R, et al: Inhibbition of hematotopoietic stem cell proliferation by hemoreggulatory peptidede pyroGlu-Glu-Asp-Cys-Lys (pEEDCK) pprovides protecttction against short-term neutropenia and lonng-term damagage. *Ann NY Acad Sci* 1991;628:92–104.
32. Cashmaman J, Eaves A, Sarris A, et al: Evidence for a novel inhibitoitor acting in normal longterm human marrow cultureres, induced by AcSDKP and inactive on pprimitive CML pi progenitors. *Blood* 1994;84:277a.
33. Springeger TA: Adhesion receptors of the immune system. *Nature re* 1990;346:425–34.
34. Teixidcdo J, Hemler ME, Greenberger JS, et al: Roole of b_1 and b_2 b_2 integrins in the adhesion of human CD34^{hi} stem cells to to bone marrow stroma. *J Clin Invest* 1992;90:5358–67.
35. Liesveleld JL, Winslow JM, Frediani KE, et al: Expreession of integrirrins and examination of their adhesive funaction in normanal and leukemic hematopoietic cellss. *Blood*

1993;81:112–21.
36. Roberts R, Gallagher J, Spooncer E, et al: Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. *Nature* 1988;332:376–8.
37. Long MW: Blood cell cytoadhesion molecules. *Exp Hematol* 1992;20:288–301.
38. Pytela R, Pierschbacher MD, Ruoslahti E: Identification and isolation of a 140 kD cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell* 1985;40:191–8.
39. Argraves WS, Suzuki S, Arai H, et al: Amino acid sequence of the human fibronectin receptor. *J Cell Biol* 1987;105:1183–90.
40. Hemler ME, Huang C, Takada Y, et al: Characterization of the cell surface heterodimer VLA-4 and related peptides. *J Biol Chem* 1987;262:11478–85.
41. Hemler ME, Elices MJ, Parker C, et al: Structure of the integrin VLA-4 and its cell-cell and cell-matrix adhesion functions. *Immunol Rev* 1990;114:45–65.
42. Patel VP, Lodish HF: The fibronectin receptor on mammalian erythroid precursor cells: characterization and developmental regulation. *J Cell Biol* 1986;102:449–56.
43. McNiece IK, Bertoncello I, Kriegler AB, et al: Colony-forming cells with high proliferative potential (HPP-CFC). *Int J Cell Cloning* 1990;8:146–60.
44. Vuillet-Gaugler MH, Breton-Gorius J, Vainchenker W, et al: Loss of attachment to fibronectin with terminal human erythroid differentiation. *Blood* 1990;75:865–73.
45. Rosemblatt M, Vuillet-Gaugler MH, Leroy C, et al: Coexpression of two fibronectin receptors, VLA-4 and VLA-5, by immature human erythroblastic precursor cells. *J Clin Invest* 1991;87:6–11.
46. Sadahira Y, Yoshino T, Monobe Y: Very late activation antigen 4-vascular cell adhesion molecule 1 interaction is involved in the formation of erythroblastic islands. *J Exp Med* 1995;181:411–5.
47. Williams DA, Rios M, Stephens C, et al: Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. *Nature* 1991;352:438–41.
48. Papayannopoulou T, Nakamoto B: Peripheralization of hemopoietic progenitors in primates treated with anti-VLA_4 integrin. *Proc Natl Acad Sci USA* 1993;90:9374–8.
49. Verfaillie CM, McCarthy JB, McGlave P: Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alterations in their interaction with fibronectin. *J Exp Med* 1991;174:693–703.
50. Simmons PJ, Masinovsky B, Longenecker BM, et al: Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. *Blood* 1992;80:388–95.
51. Verfaillie CM, Benis A, Iida J, et al: Adhesion of committed human hematopoietic progenitors to synthetic peptides from the C-terminal heparin-binding domain of fibronectin: cooperation between the integrin a4b1 and the CD44 adhesion receptor. *Blood* 1994;84:1802–11.
52. Diamond MS, Springer TA: The dynamic regulation of integrin adhesiveness. *Curr Biol* 1994;4:506–17.
53. Dialynas DP, Shao L-E, Hinojosa AG, et al: Identification and partial characterization of a novel negative regulator of hematopoiesis derived from a new human cell line. *submitted* 1995.
54. Carlos TM, Schwartz BR, Kovach NL, et al: Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells. *Blood* 1990;76:965–70.
55. Ohh M, Smith CA, Carpenito C, et al: Regulation of intercellular adhesion molecule-1 gene expression involves multiple mRNA stabilization mechanisms: effects of interferon-gamma and phorbol myristate acetate. *Blood* 1994;84:2632–9.
56. Kovach NL, Lin N, Yednock T, et al: Stem cell factor modulates avidity of a_4b_1 and a_5b1 integrins expressed on hematopoietic cell lines. *Blood* 1995;85:159–67.
57. Kovach NL, Carlos TM, Yee E, et al: A monoclonal antibody to b_1 integrin (CD29) stimulates VLA-dependent adherence of leukocytes to human umbilical vein endothelial cells and matrix components. *J Cell Biol* 1994;116:499–509.
58. Tazaka Y, Albelda SM, Horgan KJ, et al: CD31 expressed on distinctive T cell subsets is a preferential amplifer of b1 integrin-mediated adhesion. *J Exp Med* 1992;176:245–53.
59. Shimizu Y, van Seventer GA, Horgan KJ, et al: Regulated expression and binding of three VLA (b1) integrin receptors on T cells. *Nature* 1990;345:250–3.
60. Dustin ML, Springer TA: T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* 1989;341:619–24.
61. Kishimoto TK, Jutila MA, Berg EL, et al: Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science* 1989;245:1238–41.
62. Roberts AB, McCune BK, Sporon MB: TGF-b: Regulation of extracellular matrix. *Kidney Int* 1992;41:557–9.
63. Vale W, Hsueh A, Rivier C, et al: The inhibin/activin family of hormones and growth factor. *Handbook Exp Pharmacol* 1990;95:211–48.
64. Bowditch RD, Halloran CE, Aota S-I, et al: Integrin $a_{IIb}b_3$ (platelet GPIIb-IIIa) recognizes multiple sites in fibronectin. *J Biol Chem* 1991;266:23323–8.
65. Muench MO, Firpo MT, Moore MA: Bone marrow transplantation with interleukin-1 plus kit-ligand *ex vivo* expanded bone marrow accelerates hematopoietic reconstitution in mice without the loss of stem cell lineage and proliferative potential. *Blood* 1993;81:3463–73.
66. Shapiro F, Yao T-J, Raptis G, et al: Optimization of conditions for *ex vivo* expansion of CD34+ cells from patients with stage IV breast cancer. *Blood* 1994;84:3567–74.
67. Verfaillie CM, Catanzarro PM, Li W-N: Macrophage inflammatory protein 1a, interleukin 3 and diffusible marrow stromal factors maintain human hematopoietic stem cells for at least eight weeks *in vitro*. *J Exp Med* 1994;179:643–9.
68. Koller MR, Bender JG, Miller WM, et al: Expansion of primitive human hematopoietic progenitors in a perfusion bioreactor system with IL-3, IL-6, and stem cell factor. *Bio/Technology* 1993;11:358–63.
69. Reems JJ, Torok-Strob B: Cell cycle and functional differences between CD34+/$CD38^{hi}$ and CD34+/38^{lo} human marrow cells after *in vitro* cytokine exposure. *Blood* 1995;85:1480–7.

70. Fleming WH, Alpern EJ, Uchida N, et al: Functional heterogeneity is associated with the cell cycle status of murine hematopoietic stem cells. *J Cell Biol* 1993; 122:897–902.
71. Abkowitz JL, Persik MT, Shelton GH, et al: Behavior of hematopoietic stem cells in a large animal. *Proc Natl Acad Sci USA* 1995;92:2031–5.
72. Quesenberry, PJ, Ramshaw H, Rao S, et al: Transplantation of hemopoietic stem cells in nonmyeloablated mice. In: *Mehdi Tavassoli Memorial Symposium on Hematopoietic Stem Cells*, 1994:41.
73. Stewart FM, Crittenden RB, Lowry PA, et al: Long-term engraftment of normal and post-5-fluorouracil murine marrow into normal nonmyeloablated mice. *Blood* 1993;81:2566–71.
74. Martin PJ, Hansen JA, Storb R, et al: Human marrow transplantation: an immunological perspective. *Adv Immunol* 1987;43:379–438.
75. O'Reilly RJ: Allogenic bone marrow transplantation: current status and future. *Blood* 1983;62:941–64.
76. Auchincloss H Jr.: Xenogeneic transplantation. *Transplantation* 1988;46:1.
77. Schmidt-Wolf IGH, Dejbakhsh-Jones S, Ginzton N, et al: T-cell subsets and suppressor cells in human bone marrow. *Blood* 1992;80:3242–50.
78. Torbett BE, Picchio G, Mosier DE: hu-PBL-SCID mice: a modeel for human immune function, AIDS, and lymphhomagenesis. *Immunol Rev* 1991;124:139–64.
79. Dick J JE: Immune-deficient mice as models for human hemaatopoietic disease. *Mol Genetic Med* 1991;1:77–115.
80. McCuune JM, Kaneshima H, Krowka J, et al: The SCID-hu mousee: a small animal model for HIV infection and pathopgenesis. *Ann Rev Immunol* 1991;9:399–428.
81. Ferranri G, Rossini S, Giavazzi R, et al: An *in vivo* model of somatitic cell gene therapy for human severe combined immuunodeficiency. *Science* 1991;251:1363.
82. Lapiddot T, Pflumio F, Doedens M, et al: Cytokine stimulationn of multilineage hematopoiesis from immature humaan cells engrafted in SCID mice. *Science* 1992;255: 1137—41.
83. Yu YYIL, Kumar V, Bennet M: Murine natural killer cells and nmarrow graft rejection. *Ann Rev Immunol* 1992; 10:1899–214.
84. Pflumnio F, Lapidot T, Murdoch B, et al: Engraftment of humaan lymphoid cells into newborn SCID mice leads to graft-v-versus-host disease. *International Immunol* 1994;5: 1509–-22.
85. Noltaı JA, Hanley MB, Kohn DB: Sustained human hemaatopoiesis in immunodeficient mice by contransplantaation of marrow stroma expressing human interleukinn-3: analysis of gene transduction of long-lived progenitoors. *Blood* 1994;83:3041–51.